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Myofibroblasts and Their Role in Lung Collagen Gene Expression during Pulmonary Fibrosis

A Combined Immunohistochemical and in Situ *Hybridization Study*

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Appearance of contractile filament-laden stromal cells or myofibroblasts is a characteristic of lung fibrotic lesions. The role of these cells in fibrosis and their cytoskeletal phenotype are not fully delineated. This study was undertaken to further investigate these issues using a model of lung fibrosis. Rats were treated endotracheally with bleomycin on day 0, and their lungs examined at various time points by* in situ *hybridization for $\alpha_1(I)$ procollagen mRNA expression and by immunohistochemistry for desmin and α-smooth muscle actin expression. The results show an increase in the number of cells resembling fibroblasts and strongly positive for α-smooth muscle actin, desmin and procollagen mRNA expression in lungs of animals treated with bleomycin, with the increase being maximal between days 7 and 14 after bleomycin treatment. Two types of newly positive cells could be discerned. The first expressing α-smooth muscle actin, desmin, and procollagen mRNA was localized in active fibrotic lesions. The second expressing only α-smooth muscle actin and procollagen mRNA was localized in fibrotic submesothelial areas. Almost all of the newly reactive α-smooth muscle actin-positive cells strongly express procollagen mRNA, and they constituted most of the cells actively expressing procollagen. These findings suggest that the newly appearing myofibroblast characterized by α-smooth muscle actin and/or desmin expression may be responsible for most if not all of the increased lung collagen gene expression in pulmonary fibrosis. (Am J Pathol 1994, 145:114–125)

Previous studies have demonstrated that pulmonary fibrosis is characterized by increases in the number and volume of contractile filament-laden parenchymal cells as well as increased parenchymal contractility.[1–5] Similar cells expressing α-smooth muscle actin and referred to as myofibroblasts have also been shown to be present in wound healing and human and rat lung fibrosis.[6–12] The origin of these cells, the types of extracellular matrix they synthesize, and regulation of their contractile properties have not been fully defined.[13,14] Presence in large numbers within the fibrotic area and more recent study of lung tissues from patients with idiopathic pulmonary fibrosis provides evidence that in both Masson bodies and fibrotic foci, α-smooth muscle actin-positive fibroblast-like cells can express procollagen type I protein, suggesting that these cells play a role in the remodeling of the lung in pulmonary fibrosis.[11] Detailed and direct analysis of the importance of these cells in terms of collagen production during the fibrotic process are not provided in these studies. The α-smooth muscle actin-positive fibroblast-like cells from lungs of rats with bleomycin-induced pulmonary fibrosis also express vimentin, but not desmin (VA phenotype).[5,10] A recent study, however, shows that only fixation using Methacarn enables the immunohistochemical detection of desmin in lung tissue.[15] Hence, the lack of desmin staining in the rat studies[5,10] needs to be re-evaluated using this approach. This study has been undertaken to address some of these issues concerning the cytoskeletal phenotype of these myofibroblasts and to directly evaluate the potential of these cells to express procollagen.

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Materials and Methods

Animals and Tissue Sampling

Specific pathogen-free, male Fischer 344 rats weighing 200 to 250 g were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). These animals arrived in filtered cages and were maintained in clean animal quarters separate from other laboratory animals. Animals were randomly assigned to control or experimental groups. Pulmonary fibrosis was induced on day 0 by the endotracheal injection of 0.75 units bleomycin/100 g body weight in 300 μl sterile saline as previously described.[16,17] Control animals received sterile saline only. Animals (11 to 13 animals/group) were sacrificed on days 1, 3, 7, 14, 21, and 28 after bleomycin treatment. The lungs were rapidly dissected free of extraneous tissue, and the lungs from four to five animals/group were filled immediately with either neutral buffered formalin, pH 7.2, or methyl Carnoy's fixative (Methacarn; methanol/chloroform/glacial acetate acid, 60:30:10, v/v) injected through the trachea. After at least 16 hours of fixation, the lungs from the anterior, middle, and lower right segments as well as posterior and anterior left segments were cut with a razor blade and embedded in paraffin. The remainder of the lungs (three animals/group) from the control and experimental groups are used for Northern blotting analysis. Serial 3- to 5-μ sections of formalin-fixed tissues were prepared for *in situ* hybridization, immunohistochemistry, or combined *in situ* hybridization and immunohistochemistry. The serial sections of Methacarn-fixed tissues were used for immunohistochemistry only. The same neutral formalin fixation method was used for preparation of normal rat skin sections for use as positive control for the *in situ* hybridization analysis. The general histological appearance of tissue was assessed after routine hematoxylin and eosin staining.

In Situ *Hybridization*

In situ hybridization was performed essentially as previously described.[18–20] The following 30-mer oligonucleotide antisense probe (corresponding to amino acids 854–863) for α_1(I) procollagen was used[20,21]:

5′-AGGGCCAGTCTCAGCACGGTCACCCTTGGC-3′

The following corresponding sense probe was used for control purposes:

5′-GCCAAGGGTGACCGTGCAGAGACTGGCCCT-3′

These probes were synthesized by an automated DNA synthesizer and then purified by high-performance liquid chromatography before use. The oligonucleotide probes were labeled with ^{35}S using the 3′-end labeling method and then purified by electrophoresis on a 12% polyacrylamide gel.[18–20] The extracted probes were filtered through 0.2-μ filters, dried in a Speed Vac (Savant Instruments, Hicksville, NY), and then redissolved in 0.05 mol/L Tris-EDTA buffer, pH 7.8. Briefly, the paraffin sections were treated with 0.2 N HCl for 20 minutes, and then the slides were treated with 0.25% (v/v) acetic anhydride in triethanolamine for 10 minutes. This is followed by incubation in 2X SSC for 30 minutes at 70 C, and washing in 2X SSC at room temperature for 5 minutes. The slides were then overlayered with 40 μl of prehybridization buffer and incubated for 1 hour at room temperature. This was followed by the addition of 20 to 40 μl (10^5 cpm/μl) of the indicated radiolabeled oligonucleotide probe diluted in hybridization buffer. The slides were each covered with a coverslip pretreated with Sigmacote (Sigma Chemical Co., St. Louis, MO) and incubated at 44 C in a moist chamber. After 18 hours, the coverslips were gently removed in 2X SSC and the slides sequentially washed at room temperature in 2X SSC for 2 hours, 1X SSC for 1 hour, and 0.5X SSC for 30 minutes. This was followed by two sequential 30-minute washes at 44 C in 0.5X SSC. The slides were then dehydrated in 95% and 100% ethanol containing 0.3 mol/L ammonium acetate and air-dried. Autoradiographic detection of the hybrids was carried out by dipping in Kodak NBT-2 emulsion diluted 1:1 with distilled water containing 0.3 mol/L ammonium acetate for 3 to 5 seconds. The slides were allowed to air-dry for 1 hour and stored in a desiccated chamber at 4 C for 7 to 10 days. They were subsequently developed for 3 minutes in Kodak D-19 developer, washed in water for 1 minute, and fixed for 5 minutes in Kodak fixer. They were then stained with hematoxylin and eosin and coverslipped.

Controls for *in situ* hybridization consisted of 1) prior digestion of lung tissue with 100 μg/ml of RNAse A (Sigma) at 37 C for 45 minutes before addition of antisense probe, 2) substituting sense probe for antisense probe, and 3) using sections of normal rat skin as positive controls.

Immunohistochemistry

Immunohistochemistry was performed using the immunoperoxidase technique as previously described.[22,23] Briefly, endogenous peroxidase activity in sections was blocked with 3% H_2O_2 for 10 minutes. The primary anti-α-smooth-muscle-actin antibody, Immunoglobulin G (IgG_{2a} murine monoclonal, Boehringer-Mannheim, Indianapolis, IN), and anti-swine desmin antibody (IgG_1 murine monoclonal, DAKO, Denmark) were added at a final-dilution of 1:500, and 1:300, respectively,[23–25] and then incubated for 60 minutes. Biotinylated and affinity-purified horse anti-mouse IgG ("rat adsorbed," Vector Laboratories, Burlingame, CA) was used as the secondary antibody, and incubation was carried out for 30 minutes. This was followed by avidin-biotin amplification (ABC Elite, Vector Laboratories) for 30 minutes and incubation with the substrate, 0.1% 3′,3′-diaminobenzidine (Sigma) at room temperature for 3 to 5 minutes. Nuclear counterstain using hematoxylin for 1 minute was followed by graded sequential dehydration in ethanol. The specificity of the antibody was confirmed on separate samples of rat spleen and intestine.

Combined in Situ *Hybridization and Immunohistochemistry*

After *in situ* hybridization was performed with α_1(I) procollagen antisense probe as described above and finally washed in 0.5 × SSC, immunohistochemical staining with anti-α-smooth muscle actin antibody using avidin-biotin peroxidase was carried out as described above. The slides were then dehydrated in 95% and 100% ethanol with 0.3 mol/L ammonium acetate and dried. Emulsion and autoradiography were then undertaken as described above for *in situ* hybridization. Controls for *in situ* hybridization included predigestion with RNAse (100 µg/ml) and the substitution of the antisense with the sense probe.

Northern Hybridization Analysis

Northern hybridization has been described previously.[26,27] After dissecting out the lungs, they were promptly suspended in guanidine isothiocyanate solution and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY). After centrifugation, total RNA was purified. Equal amounts (20 µg/lane) were then electrophoresed in 1% agarose gels containing formaldehyde after uniformity of loading was confirmed by examination of the 28S and 18S RNA bands in separate minigels. After overnight transfer onto filters, they were baked, prehybridized, and then hybridized with oligonucleotide antisense probe (5′-AGTGCTGTCCTCTTCTTCACACATA) complementary to a sequence unique to the human α-smooth actin mRNA.[28] This probe has been shown to cross-hybridize with rat α-smooth muscle actin.[29] After washing the filter, autoradiograms were developed from these blots, and the autoradiograms quantitated by laser densitometry.

Morphometric Analysis

The number of α_1(I) procollagen mRNA, α-smooth muscle actin or desmin, and both procollagen mRNA and α-smooth muscle actin-positive cells was quantified by light microscopy (20× objective) using an ocular grid composed of 0.25-mm^2 squares. At least 20 randomly chosen noncontiguous and nonoverlapping squares were counted resulting in a total of at least 5 mm^2 from each slide (representing a lung segment) being counted. Five segments from each rat and a total of four to five rats/group were analyzed. For immunohistochemical data, the α-smooth muscle actin or desmin positive cells were counted only if a distinct nucleus could be identified as associated with stained cytoplasm or clear cytoplasmic projection. Counting excluded positive smooth muscle coats around airways and blood vessels including smooth muscle cells of venules and capillaries, as well as tip cells. These cells are present in control lungs and are positive for both α-smooth muscle actin and desmin. For *in situ* hybridization data, cell counting was done in similar fashion, except that cells containing more than five grains/cell were considered to be positive. Background staining revealed fewer than five grains/cell based on evaluation of control slides using the sense probe and/or pretreatment of the tissue section with RNAse. For combined *in situ* hybridization and immunohistochemical analysis, only cells having both the above features were considered as positive for both procollagen mRNA and α-smooth muscle actin expression.

Statistical Analysis

Positive cell counts were expressed as the average number of cellular profiles/mm^2 of lung tissue section per rat ± SEM. Means from lungs of bleomycin-treated animals were compared with the corresponding values obtained from control animals at the respective time points using the paired Student's *t*-test.

Results

Control Lungs

Control rat lungs show the expected pattern of immunostaining with the anti α-smooth muscle actin antibody[10] in both formalin- and Methacarn-fixed tissue. Smooth muscle coats surrounding major airways and blood vessels, thin continuous or discontinuous rings that surrounded the venules, and vascular lumina located at the junction of alveolar septa were very strongly stained by the antibody. It also stained the tip cells located at the alveolar septa adjacent to the alveolar ducts and the adventitia of some capillaries. Few cells of the alveolar septa were found to be positive (Figure 1A).

Inasmuch as immunostaining with the anti-desmin antibody was completely negative when the lungs were fixed in formalin, all studies using this antibody were undertaken only with Methacarn-fixed tissues. Results using this antibody generally showed intensity of staining and distribution comparable with that by the anti-α-smooth muscle actin antibody except for strong staining of smooth muscle coats around major blood vessels and variable but much less intense staining of the tip cells (Figure 1B). *In situ* hybridization of comparable control lung sections using the α_1(I) procollagen antisense probe showed a few scattered fibroblasts weakly expressing α_1(I) procollagen mRNA, localized exclusively in the adventitial areas surrounding the primary and tertiary bronchi and large blood vessels as shown previously.[20] Combined *in situ* hybridization for α_1(I) procollagen mRNA and immunostaining for α-smooth muscle actin showed that these weakly α_1(I) procollagen mRNA-positive cells were negative for α-smooth muscle actin (Figure 1C).

Bleomycin-Treated Lungs at Days 1 and 3

Examination of lung tissue sections from day 1 bleomycin-treated animals failed to show significant differences from control lungs with regard to

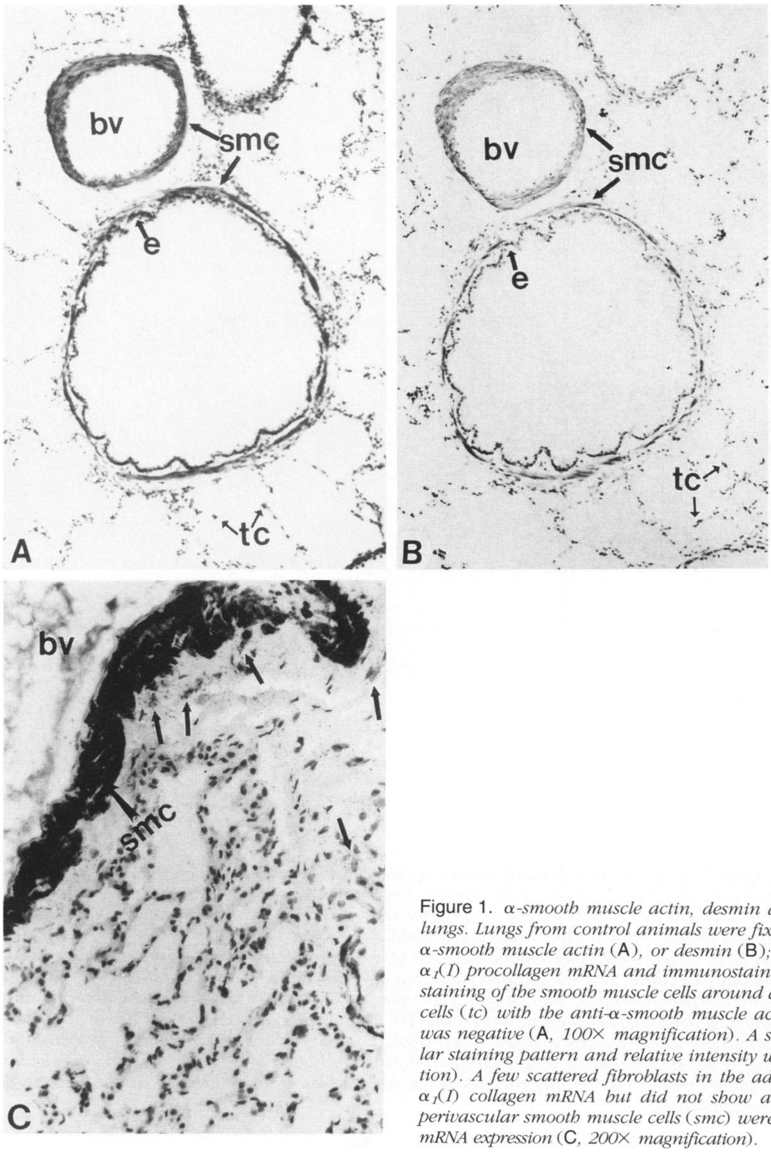


Figure 1. *α-smooth muscle actin, desmin and α_1(I) procollagen mRNA expression in control lungs. Lungs from control animals were fixed, sectioned, and stained with antibodies to either α-smooth muscle actin* (**A**), *or desmin* (**B**); *or, subjected to combined* in situ *hybridization for α_1(I) procollagen mRNA and immunostaining for α-smooth muscle actin* (**C**). *There is intense staining of the smooth muscle cells around a bronchiole and adjacent blood vessel (bv), and tip cells (tc) with the anti-α-smooth muscle actin antibody, while the bronchiolar epithelium (e) was negative* (**A**, *100× magnification*). *A serial section to the one shown in* (**A**) *shows a similar staining pattern and relative intensity with the anti-desmin antibody* (**B**, *100× magnification*). *A few scattered fibroblasts in the adventia of a blood vessel* (arrows) *were positive for α_1(I) collagen mRNA but did not show any staining with the actin antibody, whereas the perivascular smooth muscle cells (smc) were strongly actin-positive without significant collagen mRNA expression* (**C**, *200× magnification*).

α-smooth muscle actin and desmin staining, although a large number of inflammatory cells and severe pulmonary edema were present. However, significant differences between injured *versus* control lungs began to appear in day 3 bleomycin-treated rat lungs. Injured lungs revealed expanded bronchiolar adventitia or peribronchiolar (and perivascular) connective tissue, which contained increased numbers of fibroblast-like cells (based on their elongated or oval nuclei) expressing significant amounts of α_1(I) procollagen mRNA. This alteration extended to the outermost regions of some distal bronchioles and adjacent blood vessels and smooth muscle cells of venules including those located at the alveolar septal junction. However, none of these procollagen-expressing cells were positive for α-smooth muscle actin at these time points (Figure 2, A–C).

Bleomycin-Treated Lungs at Days 7 and 14

By days 7 and 14 after induction of fibrosis, significantly altered lung architecture became apparent. Peribronchial, perivascular and submesothelial inflammatory and fibrotic lesions became apparent. In some areas these extended into the alveolar space and parenchyma, resulting in expanded interstitial lesions distributed diffusely within injured lung parenchyma and replacement of edema with cellular com-

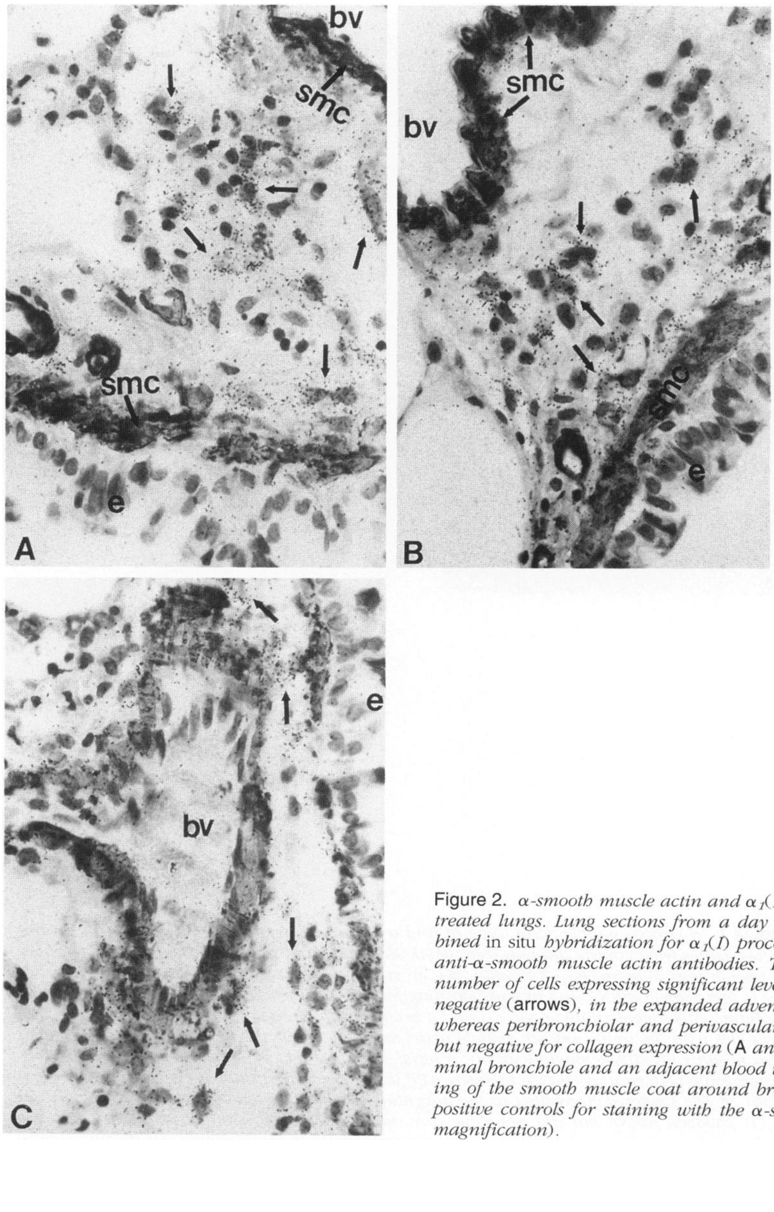


Figure 2. *α-smooth muscle actin and α_1(I) procollagen mRNA expression in day 3 bleomycin-treated lungs. Lung sections from a day 3 bleomycin-treated animal were subjected to combined* in situ *hybridization for α_1(I) procollagen mRNA expression and immunostaining with anti-α-smooth muscle actin antibodies. These day 3 lung sections show an increase in the number of cells expressing significant levels of α_1(I) procollagen mRNA but remaining actin-negative* (arrows)*, in the expanded adventitia of a bronchiole and adjacent blood vessel (bv), whereas peribronchiolar and perivascular smooth muscle cells (smc) remained actin positive but negative for collagen expression (*A *and* B*). This was also evident in the adventitia of a terminal bronchiole and an adjacent blood vessel (*C*). The relative uniform and invariant staining of the smooth muscle coat around bronchiole and blood vessels can serve as endogenous positive controls for staining with the α-smooth muscle actin and desmin antibodies (400× magnification).*

ponents. At these sites of active fibrosis, in contrast to earlier time points (and in control lungs), there was then a dramatic increase in the number of cells with high co-expression of both α_1(I) procollagen mRNA and α-smooth muscle actin (Figures 3, A–D, 4, A and B). The combined *in situ* hybridization and immunohistochemical sections showed that almost all of the newly α-smooth muscle actin-positive cells were also strongly positive for α_1(I) procollagen mRNA. In fibrotic lesions, these constitute the majority of the cells expressing α_1(I) procollagen, although a few scattered procollagen-positive cells negative for α-smooth muscle actin could be identified (Figures 3, A–D, 4, A and B).

Immunohistochemical studies of Methacarn-fixed serial sections at these two time points showed a substantial increase in the number of cells expressing desmin and α-smooth muscle actin at sites of active

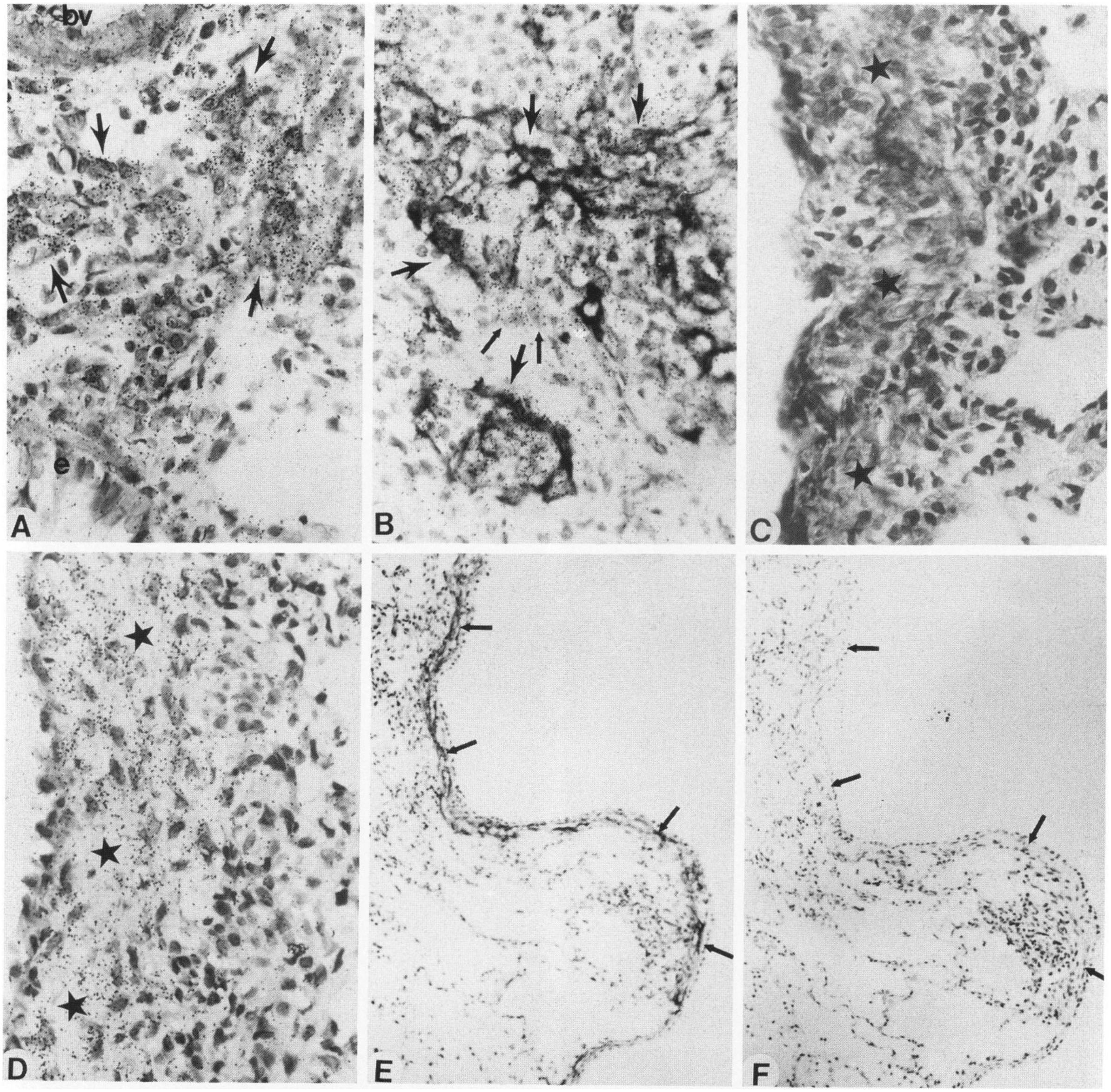
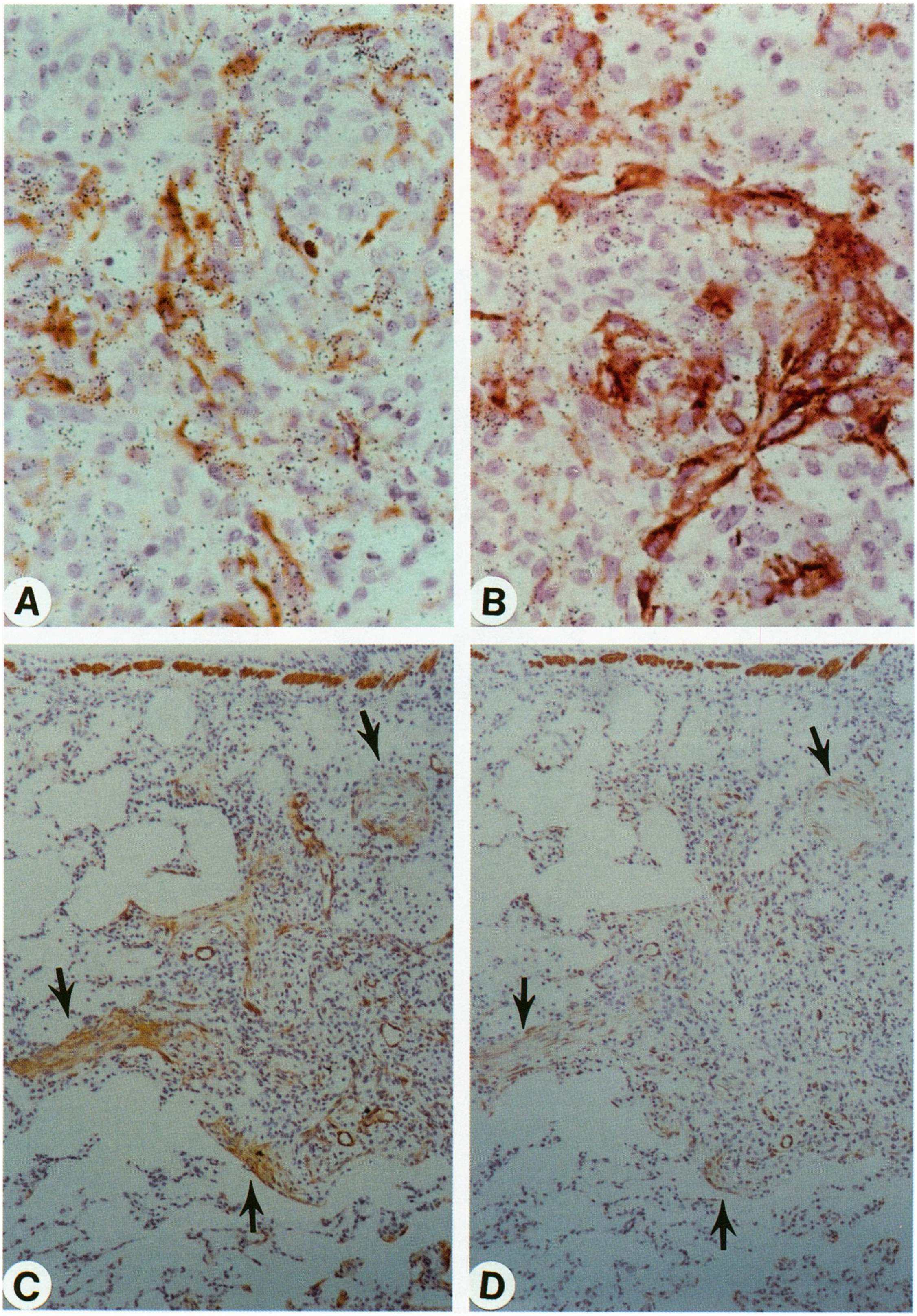


Figure 3. *α-smooth muscle actin, α_1(I) procollagen mRNA and desmin expression in day 7 bleomycin-treated lungs. Day 7 lungs were analyzed by combined* in situ *hybridization for collagen mRNA and immunohistochemistry for actin* (A *and* B), *immunohistochemistry for actin* (C *and* E) *or desmin* (F), *and* in situ *hybridization for collagen mRNA* (D). *The presence of a large number of actin-positive and collagen mRNA-expressing cells* (large arrows) *is now evident around a bronchiole and adjacent blood vessel* (A), *and within a fibrotic focus* (B). *A number of the positive collagen-expressing cells were actin-negative, however* (small arrows). *This was also evident in submesothelial fibrotic areas as shown in serial sections stained with antibody to α smooth muscle actin* (C) *and hybridized with the procollagen probe* (D), *with the actin-positive collagen-expressing cells indicated with stars. These cells of submesothelial fibrotic foci were shown to be actin-positive* (E) *but desmin-negative* (F) *in serial sections* (arrows). *Magnification was 400× except for* E *and* F, *which were 100×.*


A
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fibrosis (Figure 4, C and D). These cells usually have elongated or oval nuclei and variable numbers of cytoplasmic projections with strong cytoplasmic staining for desmin or α-smooth muscle actin. When their stained cytoplasm could be clearly outlined, some of the positive cells show fusiform or stellate-like morphology under light microscopy. The α-smooth muscle actin and desmin-positive cells tended to localize within fibrotic foci (Figure 4, C and D). Careful examination of the serial sections suggest that the desmin-positive cells in many instances are also α-smooth muscle actin-positive, or vice versa, although the desmin staining appeared to be relatively weaker (Figure 4, C and D). Antibody staining of unaffected smooth muscle coat around major airways, however, was of equal intensity for both α-smooth muscle actin and desmin (Figure 4, C and D). Most of the tip cells within these areas were hypertrophic and demonstrated enhanced staining for both α-smooth muscle actin and desmin. In other areas, however, most if not all of the strongly α-smooth muscle actin-positive cells did not express desmin. This is especially dramatic in fibrotic submesothelial areas wherein high procollagen-expressing cells were strongly positive for α-smooth muscle actin (Figure 3, C and D), but failed to stain with the anti-desmin antibody (Figure 3, E and F).

Bleomycin-Treated Lungs at Days 21 and 28

By days 21 and 28 after bleomycin treatment, the lung tissue sections revealed dramatic reduction in the number of inflammatory cells, which were replaced by irregularly shaped mature fibrotic foci that had expanded from their initial localization in the adventitia of the large airways and blood vessels as well as within submesothelial regions (data not shown). The number of α-smooth muscle actin-, desmin-, and α_1(I) procollagen mRNA-positive cells were then substantially reduced, localized primarily within mature fibrotic areas. However, a considerable number of cells remained with significant expression of procollagen mRNA but negative for α-smooth muscle actin or desmin staining.

Control negative hybridization with the sense α_1(I) procollagen probe revealed very few grains (data not shown). Hybridization of previously RNAse-digested sections with the antisense probe also revealed similarly low background.

Quantitation of Positive Cells

When cells either positive for α-smooth muscle actin, desmin, procollagen mRNA or both actin and procollagen mRNA were counted in random sections, the results confirm the above descriptive qualitative assessment. The numbers of all these cells were significantly increased after day 3, increased to a peak between days 7 and 14, and subsequently declined toward control levels by day 28 (Figure 5). The curve for the procollagen-expressing cells shows the largest increase above baseline, followed by cells positive for α-smooth muscle actin, both actin and collagen, and desmin. This is consistent with the observation that there are cells expressing collagen that are negative for actin or desmin staining. The curves for the actin-positive and the collagen mRNA-plus-actin-positive cells were almost identical, while the curve for desmin-positive cells showed a more rapid decline from a peak on day 7.

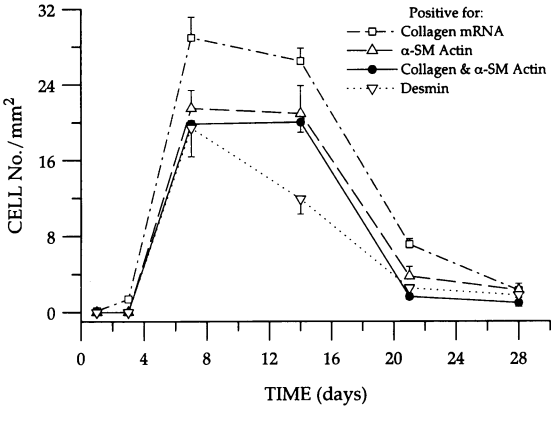

Figure 5. *Kinetics of lung cells positive for α-smooth muscle actin, desmin, α_1(I) procollagen mRNA, or both actin and procollagen mRNA. Lung sections from the indicated time points were analyzed by immunohistochemistry for actin or desmin expression, by* in situ *hybridization for collagen mRNA expression, or by combined immunohistochemistry and* in situ *hybridization for actin and collagen expression in the same sections. Cells positive for the indicated cytoskeletal protein or collagen mRNA were counted from the sections as described in Materials and Methods. Data points represent means ± SE from four to five rats/time point. The actin- and desmin-positive cell counting excluded the cells in the smooth muscle coat of airways and blood vessels.*

Figure 4. *α-smooth muscle actin, α_1(I) procollagen mRNA and desmin expression in day 14 bleomycin-treated lungs. Day 14 lungs were analyzed by combined* in situ *hybridization for collagen mRNA and immunohistochemistry for actin* (A *and* B)*, or immunohistochemistry for actin* (C) *or desmin-expression* (D)*. Fibrotic foci are shown in A and B revealing large numbers of cells now showing intense staining with α-smooth muscle actin antibody, almost all of which also strongly express collagen mRNA (400× magnification). Lower-power (100×) views show similar staining pattern or distribution for actin* (C) *and desmin* (D) *expression in serial sections of fibrotic areas* (arrows)*, although the staining intensity was weaker with desmin in the fibrotic lesions despite equal intensities in staining of the airway smooth muscle coat* (top).

Northern Analysis for α-Smooth Muscle Actin Expression

In order to quantitate the kinetics and extent of the increase in α-smooth muscle actin expression, the lungs at the various indicated time points were extracted for total RNA and subjected to Northern hybridization analysis. Autoradiograms of the blots showed a 1.7 kb species that increased in intensity beginning on day 3 after bleomycin treatment. When the blots were counted for radioactivity using a two-dimensional radioactive scanning counter, the results showed that the kinetics for increased lung actin mRNA paralleled that for the increase in the number of actin-positive cells evaluated by immunohistochemistry (Figure 6). The increase in mRNA peaked on day 14 and subsequently declined to control levels by day 30.

Discussion

Previous studies using immunohistochemistry and electron microscopy have provided indirect evidence that α-smooth muscle actin-positive myofibroblasts present in lung fibrotic lesions can synthesize procollagen and thus play an important role in the deposition of excess collagen characteristic of fibrosis.[1–12] However, direct evidence of such a role is lacking, and the evidence for desmin expression by these cells is equivocal. A related unanswered question is the origin of these actin-expressing cells.[13,14]

The results of this study for the first time provide direct evidence that at the peak of lung collagen gene expression in bleomycin-induced pulmonary fibrosis the cells primarily responsible for this increased expression were myofibroblasts as defined by expression of α-smooth muscle actin. The evidence is provided by the combined *in situ* hybridization (for procollagen mRNA expression) and immunohistochemical (for actin expression) analyses undertaken on the same tissue section, which showed co-distribution of the collagen mRNA signal with the actin staining at sites of active fibrosis. At the peak of collagen expression on days 7 and 14, almost all of the α-smooth muscle actin-positive cells in fibrotic areas were actively expressing procollagen mRNA. These cells constitute the majority of the cells expressing collagen at the peak of lung collagen gene expression.[10,20] This conclusion is also supported by the similarity in the kinetics of increase in cells positive for actin or collagen mRNA, or both. The peak of increase in newly immunoreactive actin staining coincided with that for α-smooth muscle actin mRNA, suggesting that the reactive fibroblast-like cells of days 7 and 14 may be responsible for the increased mRNA. The fact that a large amount of α-smooth muscle actin protein was detected by immunohistochemistry but not by Northern hybridization in both control and early bleomycin treatment (days 1 and 3) suggests that normal smooth muscle cells (or before any significant stimulation) around major airways and blood vessels are not actively transcribing this form of actin.

Newly desmin-positive fibroblast-like cells were also found in days 7 and 14 lungs. In view of the general similarities of their peak kinetic appearance and cellular/tissue distribution during the lung fibrotic process, most of these cells appeared to be the same ones also newly expressing α-smooth muscle actin,

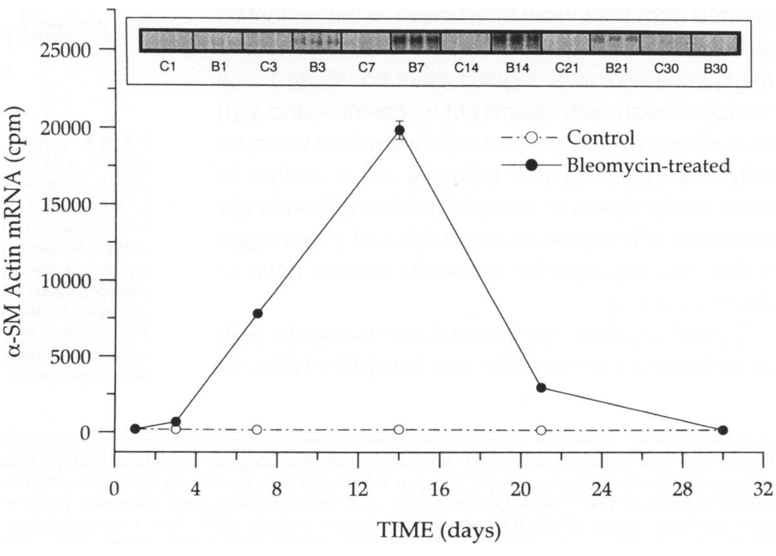

Figure 6. *Northern blotting analysis of lung α-smooth muscle actin mRNA expression. At the indicated time points, lungs from control (c) and bleomycin-treated (b) rats were extracted for total RNA and subjected to Northern analysis for determination of α-smooth muscle actin mRNA as described in methods. The autoradiogram of the blot and the results of direct quantitation by radioactive-imaging analysis of the blot are shown. Each lane in the autoradiogram represents RNA sample from a single animal. The time points refer to the number of days after bleomycin treatment on day 0. The plotted data represent means ± SE of results from three animals at each time point.*

at least as judged from serial sections of days 7 and 14 bleomycin-treated animals. The major and dramatic exception is at the submesothelial fibrotic lesions, where the cells were actin-positive, but desmin-negative. These results regarding desmin expression differ from those of previous studies showing actin- and vimentin-positive but desmin-negative cells (VA phenotype) in the same animal model.[5,10] Although the exact reason is undetermined, this apparent difference may be due to the different fixation methods used (alcohol *versus* Methacarn used in this study), and possibly the difference in time point in which the lung tissue was examined (day 28 only *versus* the earlier time points for this study). Results from a recent study demonstrate that only Methacarn fixed and paraffin-embedded lung tissues are suitable for immunohistochemical detection of desmin thus supporting the former, namely that the difference is due to different fixation techniques.[15] Data from the present study also revealed that only a few desmin-positive cells remained in lungs from day 28 bleomycin-treated animals.

Desmin expression seems to be restricted to myogenic cells such as cardiac, skeletal, and smooth muscle cells or cells tending toward myogenic differentiation and consequently has been used as one of the important markers for muscle cells.[25,30,31] However, nonmuscle cells such as Ito and mesangial cells can also express desmin CCl_4-induced liver fibrosis and immune complex nephritis, respectively.[29,32–34] The significance of the transient increase in desmin-expression in the present study is unclear, although recent studies suggest a pericyte origin for these cells.[35] The significance of desmin and/or actin expression with respect to the pathogenesis of fibrosis is unknown, but it appears to be a characteristic of proliferating and differentiating skeletal and smooth muscle cells.[30,31,35–39]

There are a number of possibilities for the multiple origin and induction of the α-smooth muscle actin- and desmin-positive cells or myofibroblasts. The smooth muscle coat surrounding airway and blood vessels appears to be an unlikely source of these newly appearing cells given that they consistently do not express much if any α_1(I) procollagen mRNA. The relative intensity and pattern of immunohistochemical staining for α-smooth muscle actin and desmin in these areas are also consistently unchanged throughout the entire fibrotic process. Pericytes (vimentin and α-smooth muscle actin-positive, desmin-negative, VA phenotype),[14,15] and contractile interstitial cells (VD phenotype)[14,15,40] have been suggested as the precursor cells from which these myofibroblasts are derived during fibrosis.[10,40] The most recent study in the same model of lung fibrosis suggests that the contractile interstitial cell is a likely candidate because of the septal location of the first α-smooth muscle actin-positive cell. Although this possibility cannot be ruled out by the data from this and previous studies, it is not fully consistent with the early appearance of high collagen-expressing cells in the adventitial areas surrounding major airways and blood vessels. These cells appear to be distinct from the pre-existing adjacent pericyte and the more distant contractile interstitial cells in the septal regions on the basis of lack of significant actin- and desmin-staining at these early (before day 7) time points. Although direct evidence is lacking, as there is no satisfactory way of tracking cells *in vivo*, it is tempting to speculate that it is these cells that subsequently became desmin- and/or actin- positive under the influence of certain cytokines or other mediators known to be produced at the later time points. This possibility is supported by recent studies demonstrating the ability to regulate α-smooth muscle actin and desmin expression by certain cytokines.[35,41–42]

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